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The Tumor Suppressors Brat and Numb Regulate Transit-Amplifying Neuroblast Lineages in *Drosophila*

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SUMMARY

In both vertebrates and insects, neurons typically arise from neural stem cells or terminally dividing intermediate progenitors. Here, we describe another mode of neurogenesis where neural stem cells generate secondary precursors that undergo multiple rounds of self-renewing transit-amplifying divisions. We identify the Posterior Asense-Negative (PAN) neuroblasts, which do not express the transcription factors Asense or Prospero. PAN neuroblasts rely on the segregating determinants Numb and Brat to generate smaller, secondary neuroblasts that in turn give rise to ganglion mother cells (GMCs) and neurons throughout larval development. In *brat* or *numb* mutants, misspecified secondary neuroblasts are unable to produce differentiated progeny and initiate tumor-like overgrowth. In *prospero* mutants, however, tumors arise from GMCs while secondary neuroblasts are correctly specified. Our data describe a transit-amplifying lineage in the *Drosophila* nervous system and suggest that different vulnerabilities in intermediate cell types can affect the outcome of tumor suppressor loss in stem cell lineages.

INTRODUCTION

The development of the *Drosophila* central nervous system has become the subject of intensive investigation as a model for the regulation of self-renewal in stem cell lineages (Chia et al., 2008). Neuroblasts are specified in the embryo, and they begin dividing in a self-renewing manner to produce neurons used by the larva. In larval and pupal stages, the divisions continue and produce the neurons of the adult fly. It has long been accepted that all neuroblasts express the neural precursor gene *asense* (*ase*) (Brand et al., 1993; Jarman et al., 1993) and divide asymmetrically to self-renew and produce a small daughter cell, the ganglion mother cell (GMC). The GMC divides terminally into two neurons or glia. During each neuroblast division, an axis of polarity is established by the activity of the Par complex, a conserved protein complex consisting of Par-3/Bazooka (Schober et al., 1999; Wodarz et al., 1999), Par-6 (Petronczki and Knoblich, 2001), and atypical Protein Kinase C (aPKC) (Rolls et al., 2003; Wodarz et al., 2000). The Par complex has two major functions. The first function is to recruit a protein called Inscuteable (Insc), which maintains the polarity of the Par complex and thereby the polarity of the neuroblast (Schober et al., 1999; Wodarz et al., 1999). Insc also directs the mitotic spindle to align along the axis of polarity (Kraut et al., 1996). The second function of the Par complex is to promote the localization of cell fate determinants to the opposite pole of the neuroblast (Betschinger et al., 2003). The cell fate determinants segregate exclusively into the GMC at telophase and act to specify GMC fate. They include the Notch repressor Numb (Knoblich et al., 1995), the transcription factor Prospero (Pros) (Hirata et al., 1995; Knoblich et al., 1995), and the NHL-domain protein Brain tumor (Brat) (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006c). In the GMC, Numb, Pros, and Brat are all thought to inhibit self-renewal and promote cell cycle exit and differentiation. Numb probably does this by promoting endocytosis of the Notch receptor, making levels of Notch signaling lower than those in the neuroblast (Berdnik et al., 2002; Wang et al., 2006a). Pros enters the GMC nucleus after degradation of its cortical anchor protein Miranda (Mira), represses expression of cell cycle genes, and activates genes required for terminal differentiation (Choksi et al., 2006; Ikeshima-Kataoka et al., 1997; Li and Vaessin, 2000; Shen et al., 1997). Brat may act to prevent cell growth (Betschinger et al., 2006; Frank et al., 2002). Consistent with the functions of these genes in repressing growth and self-renewal, loss of *brat*, *numb*, or *pros* in the larva results in neuroblast lineages that escape differentiation. This causes overgrowth characterized by the overproduction of neuroblast-like cells at the expense of differentiated neurons (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a, 2006c; Wang et al., 2006a). Although brain tissues mutant for *numb*, *pros*, or *brat* all share similar terminal phenotypes, the precise cellular events initiating the overgrowth are unknown. Close analysis of the *brat* phenotype indicates that the overgrowing cells arise in a specific location in the central brain (Bello et al., 2006; Betschinger et al., 2006), suggesting that some cells are particularly sensitive to loss of *brat*. In this study, we demonstrate that these cells comprise

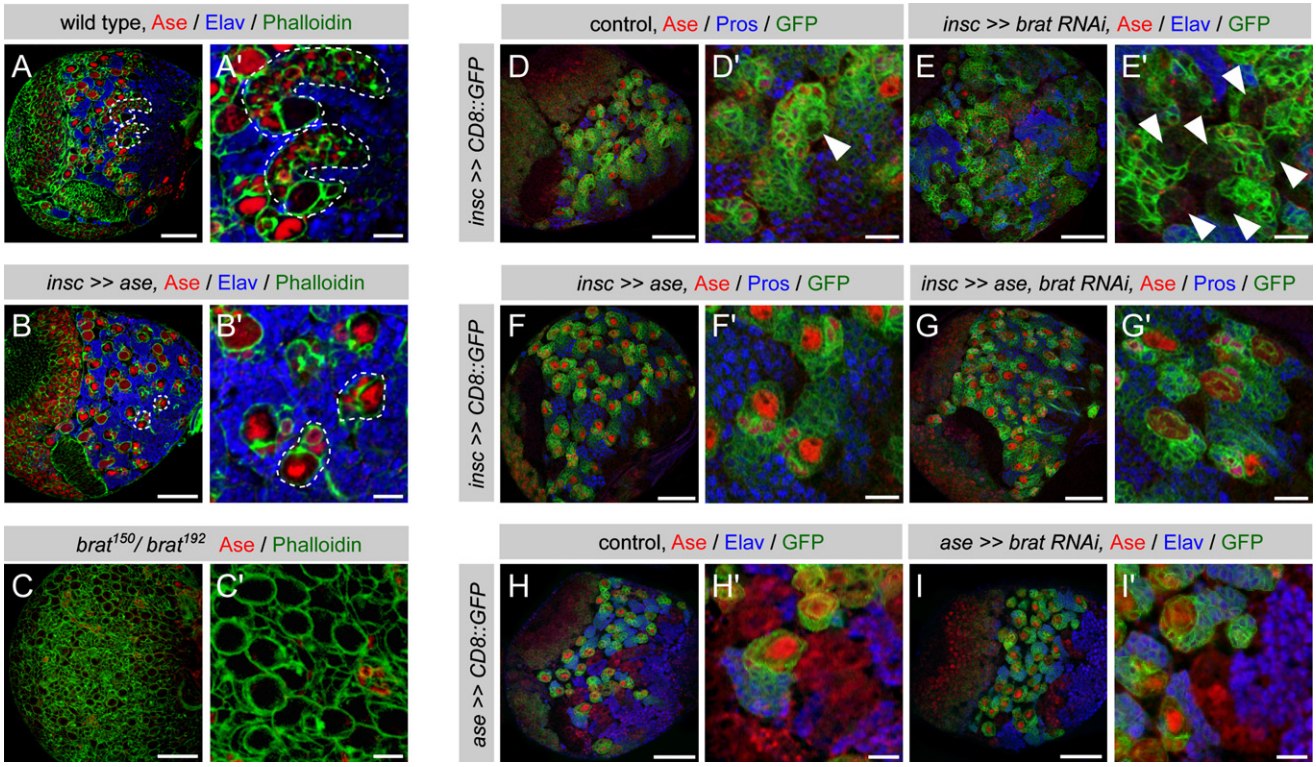

Figure 1. *brat* Overgrowth Originates in PAN Neuroblast Lineages

Immunostainings of third-instar brains labeled with indicated markers (gray boxes). For each genotype, an overview of the brain lobe and a detail of neuroblast lineages are shown.

(A and B) Wild-type brains contain PAN neuroblasts with long lineages of Ase$^+$ progeny ([A] and [A′], outline); ectopic expression of Ase eliminates both ([B] and [B′], outline).

(C and C′) A *brat* zygotic mutant brain is overgrown with PAN neuroblasts.

(D–G) Brains with GFP reporting *insc*-Gal4 expression. Control brains are well organized and contain PAN neuroblasts ([D] and [D′], arrowhead). Brat knockdown results in disorganized overgrowth with ectopic PAN neuroblasts ([E] and [E′], arrowheads). Expressing Ase in all neuroblasts eliminates PAN neuroblasts (F and F′). Ectopic expression of Ase prevents the overgrowth normally caused by Brat knockdown (G and G′).

(H and I) Brains with GFP reporting *ase*-Gal4 expression. Neither control brains (H and H′) nor Brat knockdown brains (I and I′) show disorganized overgrowth phenotypes.

Scale bars: (A)–(I), 50 μm; (A′)–(I′), 10 μm.

a previously uncharacterized neuroblast lineage with a transit-amplifying pool of intermediate progenitors. Unlike any known *Drosophila* neuroblasts, the neuroblasts generating this lineage repress Ase. We show that Brat and Numb act to promote maturation of intermediate progenitors. In the absence of these proteins, maturation fails to take place, immature progenitors begin to divide, and their progeny do not differentiate. Our data suggest that mitosis in an immature intermediate progenitor can initiate tumorous overgrowth.

RESULTS

brat Overgrowth Originates in Ase$^-$ Neuroblast Lineages

Mutation in *brat* leads to dramatic overproduction of neuroblasts at the expense of neurons (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006c). The phenotype is thought to arise from misregulated neuroblast division, but not all central brain neuroblasts are equally affected by loss of Brat (Bello et al., 2006; Betschinger et al., 2006). To find molecular markers for the sensitive subpopulation, we examined known neuroblast markers and checked for differential expression among small groups of central brain neuroblasts. The expression pattern of the transcription factor Ase fit this profile and we selected it for further analysis. Ase is a member of the *achaete-scute* complex (AS-C), a quartet of genes involved in specifying neural precursor cells. Expression of Ase typically begins after the remaining three members of the AS-C have acted together with other genes to specify neural precursor fate, and it persists after the precursor starts to divide (Brand et al., 1993).

Ase protein is present in the majority of central brain neuroblast nuclei, but absent from eight neuroblasts per brain lobe (Figure S1, see the Supplemental Data available with this article online). Due to their position on the posterior side of the brain, we refer to these neuroblasts as Posterior Asense-Negative (PAN) neuroblasts. They may tentatively be assigned to the dorsoposterior medial group of neuroblast lineages (Pereanu and Hartenstein, 2006) because of their location. Six medial PAN neuroblasts produce long chains of Ase$^+$ progeny cells with high levels of cortical actin, while Ase$^+$ neuroblasts typically have a small number of closely associated Ase$^+$ progeny (Figure 1A).

Two additional PAN neuroblasts generate progeny that populate more interior brain regions (Figure S1, and data not shown). Because they are more abundant and easier to identify, we focused our analysis on the medial PAN neuroblasts.

Since Ase is thought to be expressed in all *Drosophila* neural precursor cells after their specification from the neuroepithelium (Brand et al., 1993), as well as in the neural precursors of other insects (Wheeler et al., 2003), its absence in a subset of neuroblasts is surprising. To test whether downregulation of Ase in the PAN neuroblasts allows production of the long chains of Ase^+ progeny, we ectopically expressed Ase in all neuroblasts. For this, we used the Gal4 line 1407 inserted in the *insc* promoter (Betschinger et al., 2006), hereafter referred to as *insc*-Gal4. Ectopic Ase expression eliminates all Ase^- neuroblasts and all lineages with long chains of Ase^+ progeny containing high levels of cortical actin (Figure 1B). Since high levels of Ase could potentially interfere with the specification of neuroblast identity during embryogenesis, we prevented this by using the temperature-sensitive Gal4 inhibitor $Gal80^{ts}$ to limit Ase overexpression to the larval stages. This also eliminated PAN neuroblast lineages (PAN lineages; data not shown). We conclude from these experiments that ectopic expression of Ase eliminates PAN lineages, perhaps by transforming PAN neuroblasts to Ase^+ neuroblasts that produce fewer progeny.

PAN neuroblasts are found on the dorsoposterior central brain, the region thought to cause the *brat* overgrowth phenotype (Bello et al., 2006; Betschinger et al., 2006). To investigate whether the *brat* phenotype originates in PAN lineages, we tested whether *brat* loss of function could cause brain overgrowth in their absence. For this, we overexpressed Ase with *insc*-Gal4 and simultaneously knocked down *brat* using transgenic RNAi. In control brains, PAN lineages are clearly visible in the posterior medial region (Figures 1D and 1D′). Brat knockdown significantly reduces the amount of Brat protein (Figure S2), causing disorganized overgrowth with many ectopic PAN neuroblasts (45.6 ± 6.0 PAN neuroblasts, n = 5 brain lobes; Figures 1E and 1E′). As described above, overexpression of Ase eliminates the PAN lineages (Figures 1F and 1F′). Simultaneous overexpression of Ase and *brat* knockdown does not generate either the overgrowth or the ectopic PAN neuroblasts seen with *brat* RNAi alone (0.0 ± 0.0 PAN neuroblasts, n = 6 brain lobes; Figures 1G and 1G′), even though Brat protein is reduced to similar levels (Figure S2). This indicates that PAN lineages are required for the *brat* overgrowth phenotype. If the *brat* phenotype arises in the PAN lineages only, *brat* knockdown in Ase^+ neuroblasts should have no effect. To test this, we knocked down *brat* using *ase*-Gal4. *ase*-Gal4 is expressed in all central brain neuroblasts except for the eight PAN neuroblasts (Figure S1 and Figures 1H and 1H′). Notably, the Ase^+ progeny of the PAN lineage express *ase*-Gal4 at low or undetectable levels; this may be because transcriptional control of *ase* in these cells lies outside the 2 kb genomic fragment used to make *ase*-Gal4. While knockdown of Brat with *ase*-Gal4 significantly reduces Brat protein levels (Figure S2), it does not cause overgrowth of Ase^+ neuroblasts (Figures 1I and 1I′). We conclude from these data that PAN neuroblasts are the neuroblast subpopulation affected by mutation in *brat*, and the PAN lineages produce the tumorous growth seen in *brat* mutants. Correspondingly, overgrown *brat* mutant brains consist almost entirely of Ase^- neuroblasts (Figure 1C).

PAN Lineages Produce Intermediate Progenitors

To investigate why *brat* affects Ase^- neuroblasts specifically, we analyzed PAN lineages in greater detail using the MARCM system (Lee et al., 1999). This method allows the generation of wild-type or mutant neuroblast clones that express membrane-bound GFP in an otherwise wild-type, GFP-negative background. For this analysis, we induced clones at 48 hr after larval hatching (ALH), and examined them either 24 or 48 hr later. Ase^+ neuroblast clones always contain one Ase^+ neuroblast, several Ase^+ daughter cells, and many $Elav^+$ neurons (Figures 2A and 2B). The major difference between clones that have been developing for 24 hr (24 hr clones) and clones that have been developing for 48 hr (48 hr clones) is an increase in $Elav^+$ neurons (Table 2). Occasionally, we observed a single Ase^+ progeny cell dividing symmetrically in size, always closely associated with the neuroblast (Figure 2B′). These observations are consistent with a standard neuroblast lineage, where an Ase^+ neuroblast produces an Ase^+ GMC that divides terminally to produce two $Elav^+$ neurons.

By contrast, in 24 hr PAN neuroblast clones, the neuroblast produces almost exclusively $Elav^-$ progeny (Figure 2C, Table 2). Some daughter cells are Ase^-, but most are Ase^+. In 48 hr clones, the PAN neuroblast produces more than twice as many cells as an Ase^+ neuroblast (48 hr Ase^+ clones: 57.6 ± 3.8 progeny, n = 5 clones; 48 hr Ase^- clones: 131.4 ± 2.6 progeny, n = 5 clones). Ase^- progeny are never in mitosis, but there are around nine mitotic Ase^+ progeny per clone (9.2 ± 0.6 cells per Ase^- clone, n = 4 clones)—with some dividing as many as 11 cell diameters away from the neuroblast (Figures 2D and 2D′). These observations show that a PAN neuroblast can produce more progeny than an Ase^+ neuroblast in the same time period, and in contrast to an Ase^+ lineage, the progeny of the PAN neuroblast will enter mitosis far away from their mother.

The increased number of progeny in the PAN neuroblast clones could be produced either by more frequent divisions of the PAN neuroblasts or by multiple rounds of mitosis in the Ase^+ daughter cells. To distinguish these possibilities, we calculated the mitotic index of PAN neuroblasts. We reasoned that an increased rate of PAN neuroblast division would result in an observable increase in the fraction of mitotic PAN neuroblasts. Throughout larval development, the mitotic indices of PAN neuroblasts and Ase^+ neuroblasts are similar (Table 1), so the increased number of progeny is unlikely to arise from more frequent PAN neuroblast division. These results, together with the large number of mitotic Ase^+ daughters, suggest that the Ase^+ progeny divide multiple times to produce the large numbers of cells observed in PAN neuroblast clones. This contrasts sharply with a standard neuroblast lineage, where neuroblast daughters always divide terminally. The presence of intermediate progenitors in the medial central brain would explain why this region contains high numbers of small, BrdU-incorporating cells (Ito and Hotta, 1992).

PAN Lineages Contain Transit-Amplifying Secondary Neuroblasts

How does an Ase^- neuroblast produce Ase^+ intermediate progenitors? To test whether PAN neuroblasts stochastically produce Ase^- and Ase^+ daughters, we examined PAN neuroblast clones with telophase neuroblasts. All PAN neuroblasts observed generate Ase^- daughters (n = 7 telophase neuroblasts, Figure S3A). This indicates that the Ase^+ progeny in the clone

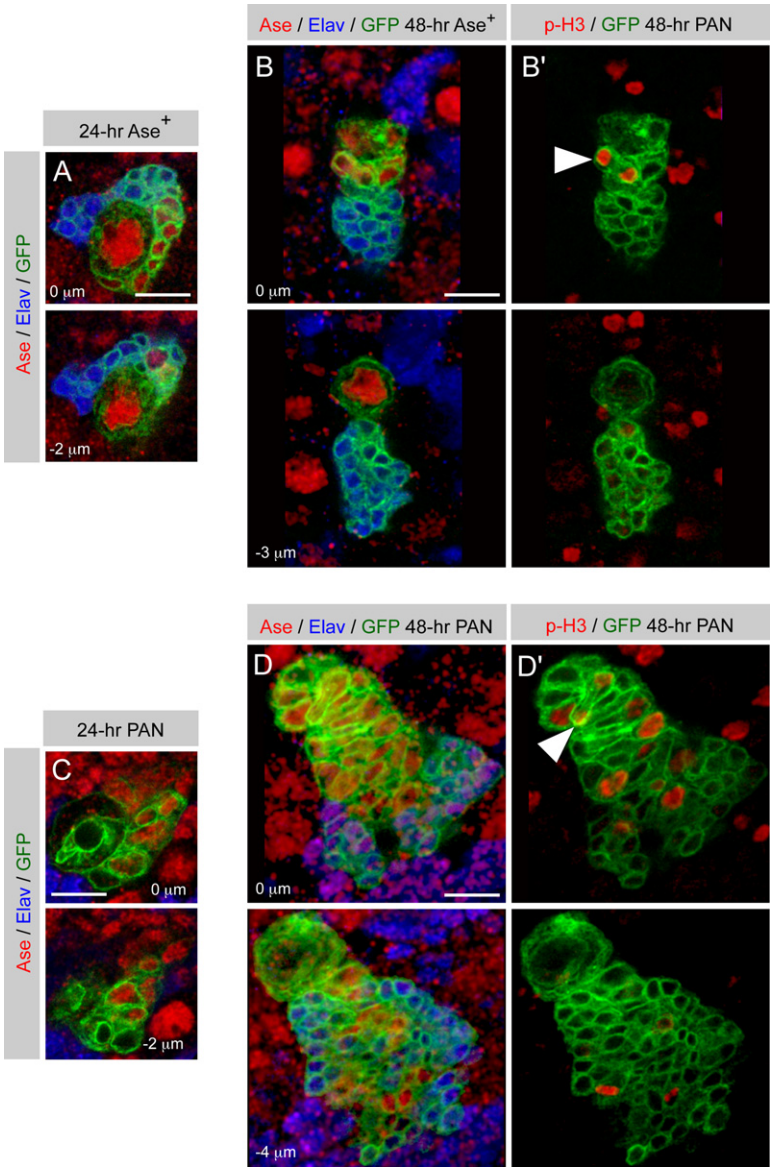

Figure 2. PAN Lineages Produce Intermediate Progenitors

Immunostainings of MARCM clones in central brain neuroblasts labeled with indicated markers (gray boxes). p-H3: phospho-histone H3. Neuroblast clones are 3D structures, so two separate optical sections from a Z-stack through each clone are shown. The most superficial section is labeled 0 μm.

(A–D) Neuroblast clones are reported by GFP expression. In 24 hr clones (A) and 48 hr clones (B), Ase$^+$ neuroblasts produce Ase$^+$ and Elav$^+$ progeny. A single Ase$^+$ cell divides symmetrically in size and adjacent to the neuroblast ([B′], arrowhead). In 24 hr PAN neuroblast clones, the neuroblast produces Ase$^-$ progeny and Ase$^+$ progeny, but almost no Elav$^+$ progeny (C). Forty-eight hour PAN neuroblast clones contain a small number of Ase$^-$ cells, and large numbers of Ase$^+$ and Elav$^+$ cells (D). All mitotic cells are Ase$^+$, several are far away from the neuroblast, and one divides asymmetrically in size ([D′], arrowhead). Scale bars: 10 μm.

must arise from these Ase$^-$ cells. We next tested whether the Ase$^-$ daughters become quiescent or remain Ase$^-$ for a long time. For this, we made use of the observation that daughter cell position correlates with time of birth: recently born daughters are near the primary neuroblast, while daughters born 1 or 2 days ago (or descendents of those daughters) tend to be several cell diameters away (compare Figures 2C and 2D). If Ase$^-$ daughters remain Ase$^-$ for longer than 24 hr, we would expect to see them far from the PAN neuroblast in 48 hr clones. We quantified this distance and found that 20% of Ase$^-$ daughters are 1 cell diameter away from the PAN neuroblast, and 80% of them directly contact it (n = 9 48 hr clones and 20 Ase$^-$ daughters). The position near the PAN neuroblast suggests that all Ase$^-$ daughters were born relatively recently, and the absence of Ase$^-$ daughters in positions occupied by older progeny indicates that Ase$^-$ status is not maintained. The most likely possibility is that these cells become Ase$^+$. These data do not directly rule out the possibility that Ase$^-$ daughters are quiescent. We therefore stained PAN neuroblast clones with anti-CyclinE (CycE). CycE marks some Ase$^-$ daughter nuclei, indicating that they are cycling cells (Figure S3B). We conclude that the PAN neuroblast produces Ase$^-$ daughters that remain Ase$^-$ for a limited period of time before becoming Ase$^+$ intermediate progenitors.

To determine the identity of the Ase$^+$ intermediate progenitors, we stained 48 hr neuroblast clones with anti-Pros and anti-Ase antibodies. Pros is present in the nuclei of GMCs and neurons but is never nuclear in neuroblasts (Bello et al., 2006; Betschinger et al., 2006). Correspondingly, in Ase$^+$ neuroblast clones, neuroblasts appear Ase$^+$Pros$^-$, GMCs appear Ase$^+$Pros$^+$, and neurons appear Ase$^-$Pros$^+$ (Figure 3A). Unlike the daughters of Ase$^+$ neuroblasts, many daughters of PAN neuroblasts do not import Pros to the nucleus. Of these daughters, two or three are Ase$^-$, and many more are Ase$^+$ (Figure 3B at 0 μm, Table 2). There are also Ase$^+$Pros$^+$ GMCs (Figure 3B at –1 μm, Table 2). Absence of nuclear Pros is consistent with a neuroblast-like cell type. To investigate this further, we checked the expression of neuroblast markers Deadpan (Dpn) and CycE in the PAN lineage. Dpn and CycE are typically confined to one or two adjacent daughters in Ase$^+$ neuroblast lineages (data not shown; Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006c). In PAN lineages, Dpn and CycE maintain high levels of expression in large numbers of progeny (Figures 3C and 3D). This implies that many of these daughters could be secondary neuroblasts.

To find out if the presumptive secondary neuroblasts divide asymmetrically, we analyzed the localization of several proteins known to regulate asymmetric divisions in embryonic and larval neuroblasts. Mitotic GMCs localize Insc to a cortical crescent, but not Mira, because Mira is degraded after GMC birth (Figures 3E and 3E′, Ikeshima-Kataoka et al., 1997; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1997). In the PAN lineages, some daughters maintain cortical Mira (Figure S4B) and, like

Table 1. Mitotic Index of Central Brain Neuroblasts

	Ase$^+$			PAN		
Stage	Total NB	Mitotic NB	Mitotic index	Total NB	Mitotic NB	Mitotic index
48 hr ALH	260	71	27%	84	18	21%
72 hr ALH	276	66	24%	81	23	28%
96 hr ALH	491	139	28%	94	20	22%

Staged larvae were stained for Ase, Phalloidin, and phospho-histone H3 to quantify the mitotic index. NB, neuroblast.

neuroblasts, localize Insc and Mira to opposing crescents in mitosis (Figure 3F′). Similar observations were made for Pros, which is dispersed in the cytoplasm of mitotic GMCs, but forms a crescent in some mitotic progeny of the PAN lineage (Figures 3G′ and 3H′). Furthermore, Brat, Numb, and aPKC all show asymmetric localization in the presumptive secondary neuroblasts (Figures S4C–S4E), and asymmetrically sized divisions occur in these cells as well (Figure 2D, arrowhead). All of these observations strongly suggest that the Ase$^+$ cells without nuclear Pros are indeed asymmetrically dividing secondary neuroblasts.

If the secondary neuroblasts divide in a self-renewing manner, they should generate MARCM clones with more than four neurons. We therefore searched for these clones in the posterior medial brain. Besides the PAN neuroblast clones, many clones in this region consist of one or two Elav$^+$ cells (data not shown), the clone type predicted by an origin in GMCs. It is also possible to observe clones in this region with multiple Ase$^+$ cells (Figure 3I) or clones with more than four Elav$^+$ cells (Figure 3J). Due to the low frequency of MARCM clone induction, it is unlikely that these multicell clones are overlapping GMC clones. Instead, they probably arise from sequential, GMC-producing divisions of the small neuroblast-like cells. Since some clones consist exclusively of multiple Elav$^+$ cells, secondary neuroblasts may eventually divide terminally or die. These findings indicate that a PAN neuroblast divides asymmetrically to produce a secondary neuroblast. The secondary neuroblast is initially Ase$^-$, but it eventually upregulates Ase and divides asymmetrically to self-renew and generate a GMC.

To test whether such a lineage could produce the observed cell types in PAN neuroblast clones, we made a computational model of the PAN lineage. The program tracked the numbers of the various cell types over time, starting with an initial division of the PAN neuroblast at time $t = 0$. This was done by looping simple, partially recursive population functions according to division rate and latency time parameters (see Supplementary Experimental Procedures for additional details). Using the model, we were able to determine a limited range of time for each event in the proposed PAN neuroblast lineage that returned values in good agreement with the observed cell populations at 24 and 48 hr (Figure 6). We conclude from this that the proposed lineage can plausibly generate the numbers of secondary neuroblasts, GMCs, and neurons observed in PAN neuroblast clones.

brat Secondary Neuroblasts Fail to Progress beyond the Immature Ase$^-$ State

To investigate the initial events leading to overgrowth in *brat* PAN lineages, we analyzed 24 hr *brat* MARCM clones. *brat* clones in Ase$^+$ neuroblast lineages do not overgrow (Figure 4B, Table 3), confirming that these lineages are unaffected by loss of Brat. Surprisingly, *brat* clones in PAN lineages show an undergrowth phenotype at 24 hr—they contain about half the number of progeny as wild-type clones (Figure 4C, Table 3). We determined the identity of the *brat* daughters by staining with anti-Pros. In wild-type PAN neuroblast clones, some cells import Pros into the nucleus (Figure 4A, Table 2), showing that after 24 hr secondary neuroblasts divide and give rise to Pros$^+$ GMCs and neurons. In *brat* PAN neuroblast clones, no progeny have nuclear Pros, suggesting that all are secondary neuroblasts (Figure 4C). While other groups report similar findings on the absence of nuclear Pros in *brat* (Bello et al., 2006; Lee et al., 2006c), we use Pros staining simply to discriminate neuroblasts from more differentiated cells. If the *brat* secondary neuroblasts entered mitosis, we would expect to see far more than the ten progeny produced in 24 hr clones. This implies a cell cycle block in *brat* secondary neuroblasts. In agreement with this, previous work shows that *brat* neuroblasts generate progeny that are cell cycle delayed (Lee et al., 2006c).

To investigate the nature of the cell cycle block, we checked the expression of cell cycle markers in the larval brain. Mitotic central brain neuroblasts express the G1-S transition markers CycE and E2F1, and these proteins associate with segregating DNA in late telophase (Figures S5A and S5B). This should cause the larval neuroblast and its daughter to enter S phase shortly after cytokinesis, similar to embryonic neuroblasts (Weigmann and Lehner, 1995). To analyze the kinetics of this in greater detail, we stained clones containing telophase PAN neuroblasts with anti-E2F1, which does not label S-phase cells, and with anti-CyclinA (CycA), a marker for S and G2 phases (Reis and Edgar, 2004). In wild-type clones, the E2F1-positive daughter being born is located near other daughters with low or undetectable levels of E2F1 (Figure S5C). Since the PAN neuroblast always directs the birth of its daughter toward a group of Ase$^-$ secondary neuroblasts (Figure S3, n = 7 telophase PAN neuroblasts), it is likely that the daughters with low E2F1 are both Ase$^-$ and recently born. The low E2F1 indicates a rapid entry into S phase by the Ase$^-$ secondary neuroblast shortly after its birth. This is followed by accumulation of CycA and re-expression of E2F1, indicating progression to G2 (Figures S5C and S5C′). Importantly, we observe the same events in *brat* secondary neuroblasts, so they must also progress through S phase (Figure S5D). Therefore, we conclude that the cell cycle delay in *brat* secondary neuroblasts occurs in G2. Since the number of progeny in the 24 hr *brat* clone is small (Table 3), the block could be maintained for around 24 hr.

In order to find out why the cell cycle is blocked in *brat* secondary neuroblasts, we analyzed other regulators of cell proliferation. The progrowth and proliferation protein dMyc is present in both wild-type and *brat* secondary neuroblasts (Figure S6), indicating that the delay is not due to lack of growth stimulus. The Cdk inhibitor Dacapo (Dap) could potentially slow the cell cycle, but we did not detect Dap in *brat* secondary neuroblasts (data not shown). To test whether the delay is caused by a defect in the differentiation of secondary neuroblasts, we checked the expression of Ase. In wild-type PAN neuroblast clones, Ase is upregulated some time after daughter cell birth (Figure 2C). In *brat* mutant PAN neuroblast clones, upregulation of Ase never occurs (Figure 4D). This suggests that the *brat* mutant secondary

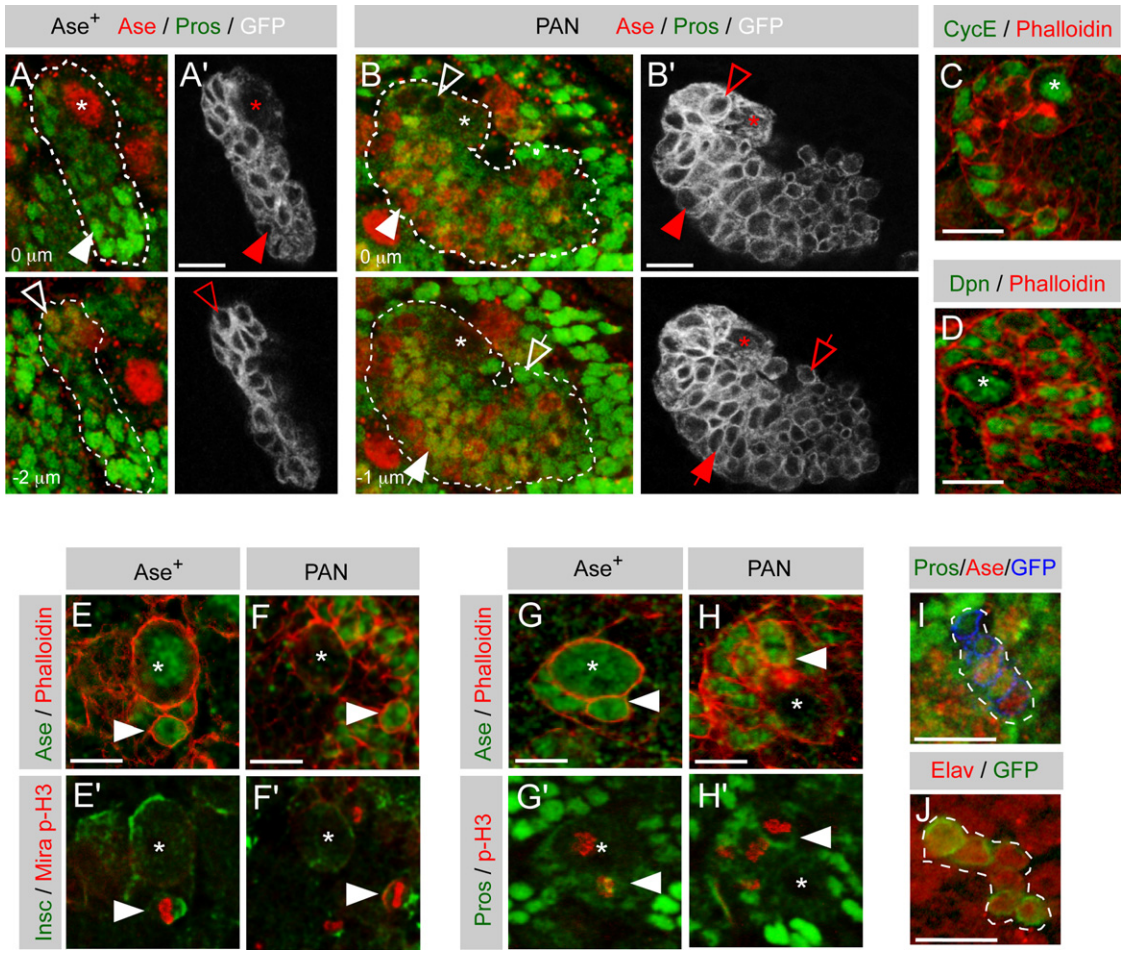

Figure 3. PAN Lineages Contain Secondary Neuroblasts

Immunostainings of larval brains labeled with indicated markers (gray boxes). Primary neuroblasts are marked with a star.

(A and B) Forty-eight hour MARCM clones in central brain neuroblasts. Two separate optical sections from a Z-stack through each clone are shown. Neuroblast clones are reported by GFP expression. (A and A′) Ase$^+$ neuroblasts exclude Pros from the nucleus, and generate Ase$^+$Pros$^+$ GMCs (open arrowhead) and Ase$^-$Pros$^+$ neurons (closed arrowhead). (B and B′) PAN neuroblasts do not have nuclear Pros. All Ase$^-$ (open arrowhead) and many Ase$^+$ (closed arrowhead) daughters do not have nuclear Pros. GMCs are Ase$^+$Pros$^+$ (closed arrow) and neurons are Ase$^-$Pros$^+$ (open arrow).

(C and D) Many PAN neuroblast progeny express the neuroblast markers CycE (C) and Dpn (D).

(E–H) Neuroblast daughter cells in mitosis (arrowheads). Mira is not present in mitotic GMCs (E′) but segregates asymmetrically in some progeny of the PAN neuroblast (F′). Similarly, Pros is cytoplasmic in mitotic GMCs (G′) but asymmetric cortical in some PAN progeny (H′).

(I and J) Twenty-four hour (I) and forty-eight hour (J) secondary neuroblast clones contain multiple Ase$^+$ cells (I) and multiple neurons (J).

Scale bars: 10 μm.

neuroblasts have not fully matured. Collectively, these observations show that loss of Brat does not impede secondary neuroblast entry into S phase, but it does prevent upregulation of Ase. This triggers a G2 block in the Ase$^-$ secondary neuroblast.

Immature Ase$^-$ Secondary Neuroblasts Enter Mitosis in *brat*

A terminal cell cycle delay in an immature secondary neuroblast would not produce overgrowth in *brat*. Therefore, we checked whether the Ase$^-$ secondary neuroblasts ever entered mitosis in *brat* clones. In wild-type PAN lineages, Ase$^-$ secondary neuroblasts are never positive for phospho-histone H3 (Figures 2D and 2D′, and data not shown). In 24 hr clones in *brat* PAN lineages, we occasionally observe Ase$^-$ secondary neuroblasts in mitosis (Figure 4D′). Although all cells in the clone are Ase$^-$, it is possible to distinguish secondary neuroblasts from PAN neuroblasts by size: the PAN neuroblast has a cell diameter greater than 10 μm, while secondary neuroblasts have a diameter of around 5–7 μm. Mitosis in an immature secondary neuroblast with a deregulated cell cycle may be the event that initiates *brat* mutant overgrowth. By the 48 hr time point, mitotic Ase$^-$ cells have increased in size and number (Figure 4E). Most cells in the *brat* clone remain Ase$^-$, showing that the daughters of the mitotic Ase$^-$ cells also fail to upregulate Ase and differentiate.

Could the mitotic immature secondary neuroblast be solely responsible for the *brat* overgrowth? The *brat* PAN neuroblast cannot generate the bulk of the cells, since it produces only 10 cells in 24 hr (Table 3) and the 48 hr brat clone contains over 100 cells (124 ± 14 cells, n = 5 clones). If *brat* mutant Ase$^+$ secondary neuroblasts or GMCs cause overgrowth, we should be unable

Table 2. Composition of Wild-Type Neuroblast Clones

Clone Type	n	*Ase*$^-$*Elav*$^-$ (2° NB)	*Ase*$^+$*Elav*$^-$ (2° NB and GMC)	*Ase*$^-$*Elav*$^+$ (neuron)	Divisions (GMC+neuron/2)
Ase$^+$ 24 hr	13	0	7.5 ± 0.6	20.3 ± 1.2	17.7 ± 0.7
Ase$^+$ 48 hr	5	0	5.4 ± 0.5	52.2 ± 3.6	31.5 ± 2.0
PAN 24 hr	9	3.4 ± 0.2	21.0 ± 1.6	2.1 ± 0.7	ND
PAN 48 hr	5	2.2 ± 0.2	47.0 ± 3.1	82.2 ± 4.8	ND
Clone type	n	*Ase*$^-$*nPros*$^-$ (2° NB)	*Ase*$^+$*nPros*$^-$ (2° NB)	*Ase*$^+$*Pros*$^+$ (GMC)	*Ase*$^-$*Pros*$^+$ (neuron)
PAN 24 hr	5	4.0 ± 0.3	9.4 ± 0.5	7.8 ± 1.2	2.2 ± 0.6
PAN 48 hr	4	3.3 ± 0.3	27.5 ± 1.7	22.8 ± 2.7	90.3 ± 3.8

Cell composition of MARCM clones stained for Ase and Elav (upper part) or Ase and Pros (lower part). All cells in the clone were counted and then tabulated according to the indicated marker profile. For PAN neuroblasts, we focused on the medial population. nPros, nuclear Pros. Error is standard error of the mean. NB, neuroblast. ND, not determined, because GMCs and neurons are not specifically detected.

to detect MARCM clones with more than one neuron. However, we regularly observe *brat* clones containing multiple Elav$^+$, Pros$^+$, and Ase$^+$ cells in the medial central brain (Figures 4F and 4G). This leaves the Ase$^-$ secondary neuroblast as the likely origin of the overgrowth. We conclude from these observations that the earliest events in *brat* overgrowth are failure to achieve Ase$^+$ secondary neuroblast status and cell cycle block in G2. Escape from the block and completion of mitosis in the immature Ase$^-$ secondary neuroblast establishes a lineage that is unable to produce differentiated daughter cells (Figure 4E). We propose that these events are the source of the overgrowing neuroblasts characteristic of *brat* mutant brains.

Notch Signaling Regulates Secondary Neuroblasts in PAN Lineages

We have shown that loss of Brat causes defective differentiation in transit-amplifying secondary neuroblasts and that this leads to overgrowth. It is therefore critical to understand how this transit-amplifying lineage is specified and regulated. In some mammalian transit-amplifying stem cell lineages, Notch controls proliferation and differentiation (Wilson and Radtke, 2006). Furthermore, in *Drosophila* larval brains, mutation in *numb*, an antagonist of Notch, causes overgrowth and production of ectopic neuroblasts (Lee et al., 2006a; Wang et al., 2006a). To test whether misregulation of Notch signaling causes defects in the transit-amplifying PAN lineages, we generated *numb* MARCM clones. In Ase$^+$ neuroblast lineages, *numb* loss of function does not affect production of differentiated GMCs and neurons (Figure 4H and Figure S7A). By contrast, *numb* mutant 24 hr PAN neuroblast clones contain secondary neuroblasts delayed in G2 (Figure 4I, Table 3, and Figure S5E). This phenotype is identical to that caused by loss of Brat. Similarly, 48 hr PAN neuroblast clones mutant for *numb* are filled with undifferentiated Ase$^-$ cells (Figure 4J, Figure S7B). This indicates that Numb is required to promote the maturation of Ase$^-$ secondary neuroblasts, perhaps by downregulating Notch signaling. To check whether ectopic activation of Notch generally causes overgrowth of the PAN lineage, we expressed the Notch intracellular domain (N^{intra}) in all neuroblasts using *insc*-Gal4. Because it is lethal at embryonic stages, we restricted N^{intra} expression to the larval stages using Gal80^{ts}. Consistent with previous results (Wang et al., 2006a), we observed that upon N^{intra} overexpression, the brain becomes filled with PAN neuroblasts (Figure S7D). A similar phenotype is observed when Notch is hyperactivated through loss of function of *lgd* (Figure S7E), a gene required for protein trafficking of the Notch receptor and downregulation of Notch signaling (Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). These results show that overactivation of Notch leads to the production of ectopic PAN neuroblasts, probably by interfering with differentiation in the PAN lineages.

Since Notch overactivation results in ectopic PAN neuroblasts, we tested whether Notch loss of function would result in too few PAN neuroblasts. For this, we used *insc*-Gal4, Gal80^{ts}, and transgenic Notch RNAi to deplete Notch in all neuroblasts (Figure S8). Knockdown of Notch has two different effects on PAN lineages: it either eliminates them entirely or reduces the number of associated Ase$^+$ progeny (Figure S7G). Similarly, we observe complete absence of PAN lineages when Notch signaling is inhibited by the expression of Numb in all neuroblasts (Figure S7H). We conclude from these results that Notch signaling must be active in the PAN neuroblast and the secondary neuroblasts to produce a wild-type lineage, but overactivation of Notch causes uncontrolled division of Ase$^-$ secondary neuroblasts.

Pros Acts after Brat and Numb in the PAN Lineage

Since loss of Brat or Numb causes defects only in PAN lineages, we investigated whether this is also true for the cell fate determinant Pros. The absence of nuclear Pros in *brat* clones has led to a model where Brat exerts its effects on cell fate by regulating Pros (Bello et al., 2006; Lee et al., 2006c). If this is true, then loss of Brat and loss of Pros should have comparable consequences. We tested this by generating *pros* MARCM clones. In Ase$^+$ lineages, *pros* clones fail to produce significant numbers of Elav$^+$ neurons in 77% of clones examined (n = 26 Ase$^+$ clones; Figures 5A and 5B), a phenotype distinct from *brat* or *numb*. In the PAN lineages, loss of Pros results in production of many Ase$^+$ progeny but almost no Elav$^+$ cells (Figures 5C and 5D). These results confirm that Pros is required to make differentiated neurons (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Lee et al., 2006c). The presence of Ase$^+$ progeny in the *pros* mutant PAN lineage indicates a requirement for Pros only after the transition from Ase$^-$ to Ase$^+$ secondary neuroblast status. We conclude that Pros acts at a later time point in the PAN lineage than Brat. Consistent with this, *pros*-Gal4 is not detectably expressed in PAN neuroblasts or Ase$^-$ secondary neuroblasts, and it becomes visible only in Ase$^+$ secondary neuroblasts (Figures 5E and 5F). We cannot exclude the possibility

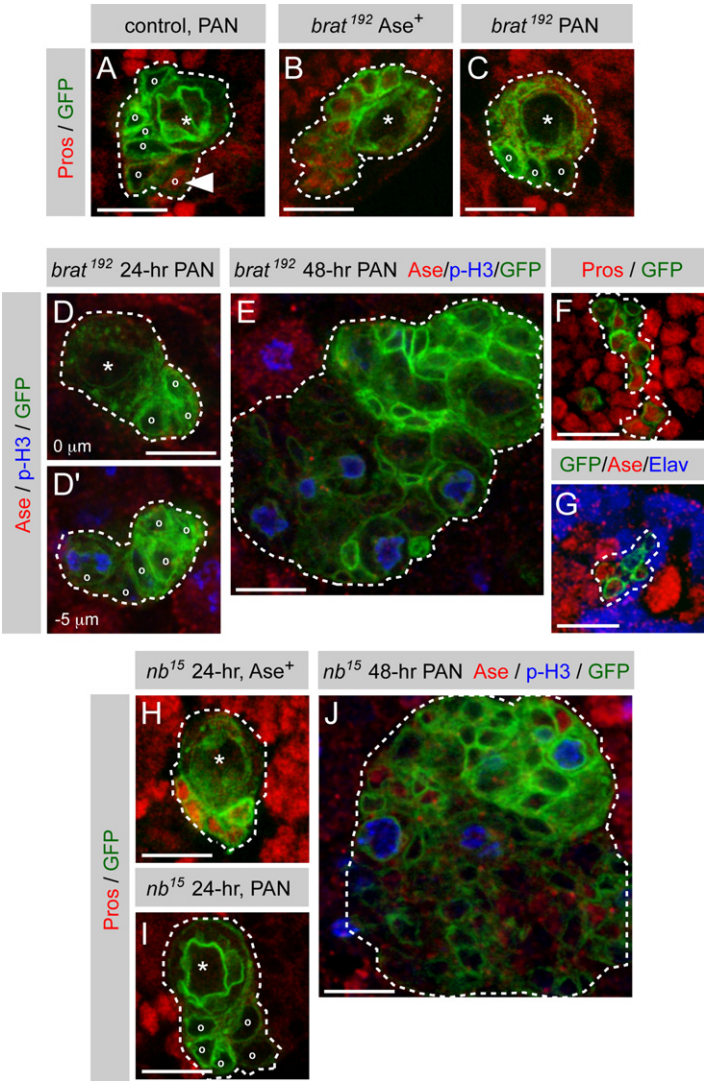

Figure 4. Immature Ase⁻ Secondary Neuroblasts Enter Mitosis in *brat*

Immunostainings of MARCM clones reported by GFP expression and labeled with indicated markers (gray boxes). Stars indicate primary neuroblasts and circles indicate daughter cells.

(A–C) Pros in 24 hr clones. In wild-type PAN neuroblast clones, Pros is nuclear in some daughters ([A], arrowhead). In *brat* Ase$^+$ clones, Pros is nuclear in all daughters (B), but in *brat* PAN neuroblast clones, no daughters have nuclear Pros (C).

(D and E) Mitotic Ase$^-$ cells in *brat* PAN neuroblast clones. In 24 hr clones, Ase$^-$ secondary neuroblasts enter mitosis (D′). In 48 hr clones, mitotic Ase$^-$ cells increase in size and number (E).

(F and G) *brat* secondary neuroblast clones. Differentiated Pros$^+$ cells (F) and Ase$^+$ and Elav$^+$ cells (G) are created in the absence of *brat*.

(H–J) *numb* clones. In 24 hr *numb* Ase$^+$ clones, all daughters have nuclear Pros (H). Pros is not nuclear in daughters of *numb* PAN neuroblasts (I). Ase$^-$ daughters enter mitosis in *numb* 48 hr PAN neuroblast clones.

Scale bars: 10 μm.

that *pros* transcription in the PAN neuroblasts and Ase$^-$ secondary neuroblasts requires a promoter not contained in *pros*-Gal4. Still, since loss of Brat and loss of Pros cause fate misspecification in different cell types, these results demonstrate that Brat does not act exclusively by regulating Pros.

The different sensitivities of Ase$^+$ and PAN neuroblasts to loss of cell fate determinants prompted us to analyze whether brain overgrowth caused by other genetic lesions begin in PAN lineages. Overexpression of membrane-targeted aPKC (aPKC^{CAAX}) (Lee et al., 2006b) and loss of the mitotic kinase *aurora-A* (AurA) (Lee et al., 2006a; Wang et al., 2006a) cause overgrowth in both Ase$^+$ and PAN lineages (Figures 5I and 5J), but loss of *lgl* (Betschinger et al., 2006) affects PAN lineages much more strongly than Ase$^+$ lineages (Figure 5K). This may be because aPKC^{CAAX} and *aurA* affect the function of proteins that promote the differentiation of GMCs and secondary neuroblasts, while *lgl* misregulates proteins required for differentiation of secondary neuroblasts more severely. We conclude that while PAN lineages contribute to a general neuroblast overgrowth phenotype in the aPKC^{CAAX} and *aurA* brains, they are primarily responsible for the overgrowth in *lgl* mutants.

Table 3. Composition of 24 hr *brat*^{192} or *numb*^{15} Neuroblast Clones

Clone type	n	*Elav*$^-$	*Elav*$^+$	Divisions (GMC+neuron/2)
brat, Ase$^+$	10	8.3 ± 0.6	14.3 ± 2.3	15.5 ± 1.1
brat, PAN	10	10.0 ± 0.7	0.0 ± 0.0	ND
numb, PAN	11	11.5 ± 1.5	0.0 ± 0.0	ND

Cell composition of MARCM clones stained for Ase and Elav. Error is standard error of the mean. ND, not determined, because GMCs and neurons are not specifically detected.

DISCUSSION

It was previously thought that all daughters of *Drosophila* neuroblasts are GMCs. We show that several neuroblasts in the *Drosophila* larval brain repress Ase and produce daughters that behave like secondary neuroblasts. The proteins Brat and Numb act to promote the progression of recently born secondary neuroblasts from an Ase$^-$ to an Ase$^+$ status. Once the secondary neuroblast becomes Ase$^+$, it begins to divide and produce GMCs. In the absence of Brat or Numb, the transition from Ase$^-$ to Ase$^+$ secondary neuroblast fails to occur, and the Ase$^-$ secondary neuroblast enters mitosis. This incorrectly specified mutant daughter cell is unable to make differentiated progeny, and it initiates overgrowth of neuroblast-like cells in the larval brain.

Ase and Transit-Amplifying Neuroblast Lineages

Ase is best known for its role as a neural precursor gene, so its absence from any neuroblast is surprising. Three mechanisms are known to downregulate expression of Ase. One is transcriptional repression mediated by Pros (Choksi et al., 2006). Since Pros is not nuclear in neuroblasts, it is unlikely to repress *ase* in the PAN neuroblasts. The transcription factor Tramtrack (Ttk) also represses *ase* transcription (Badenhorst et al., 2002), and we analyzed the reporter line *ttk*^{0219} to see if *ttk* expression correlated with the PAN lineage. Although the *ttk* reporter is

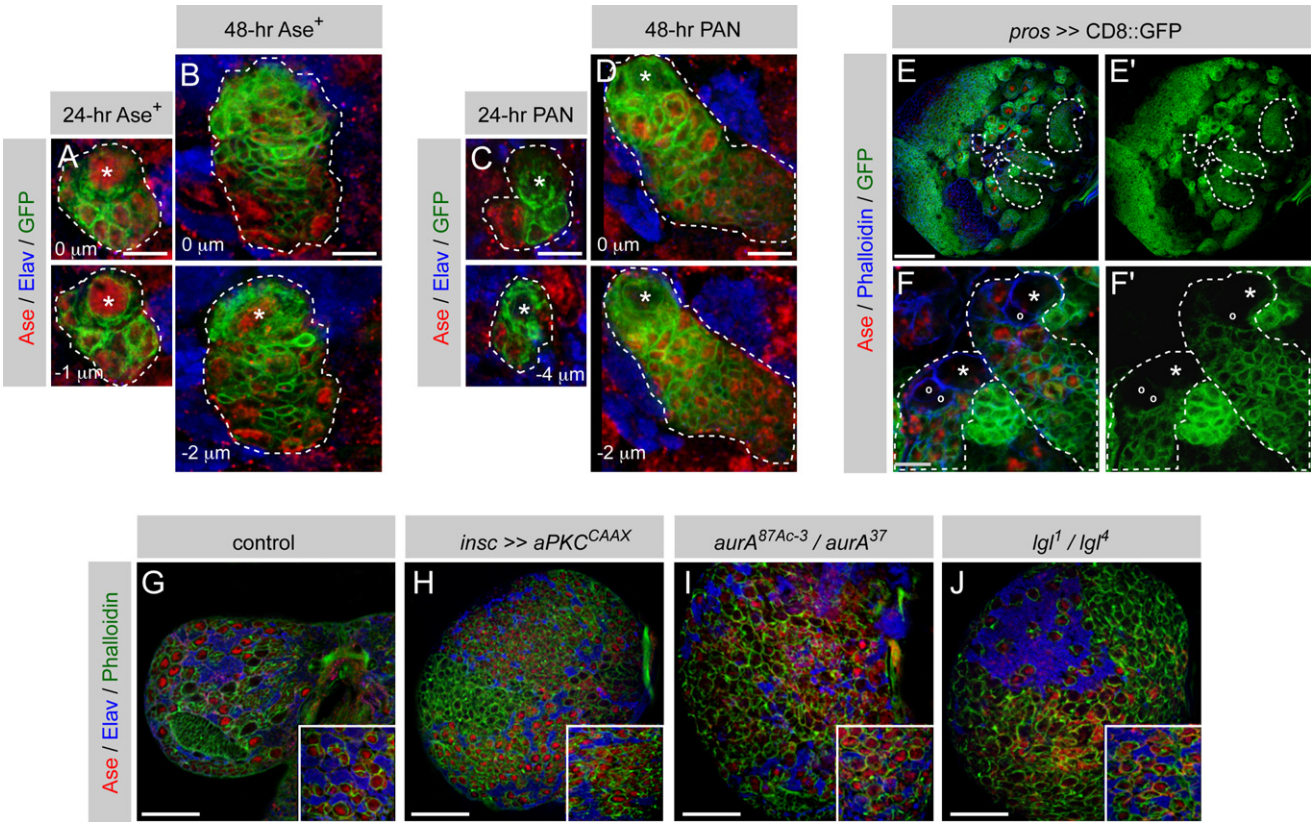

Figure 5. Pros Is Not Required for Maturation of Secondary Neuroblasts

Immunostainings labeled with indicated markers (gray boxes).

(A–D) *pros* MARCM clones reported by GFP expression. Stars indicate primary neuroblasts. Two separate optical sections from a Z-stack through each clone are shown. Loss of Pros in Ase$^+$ lineages results in failure to generate neurons (A and B). In PAN lineages, Pros is not required to generate Ase$^+$ daughters, but neurons fail to differentiate (C and D).

(E and F) *pros*-Gal4 expression reported by GFP. *pros*-Gal4 is not expressed in the PAN neuroblasts (E) or their Ase$^-$ daughters (circles, [F]).

(G–J) PAN lineage behavior in other tumor models from early third-instar larval brains. Insets: Ase$^+$ neuroblasts of the ventral nerve cord. Expression of membrane-targeted aPKC (H) or loss of AurA (I) results in overgrowth in both Ase$^+$ and PAN lineages, but loss of Lgl affects PAN lineages more strongly (J).

Scale bars: (A)–(D) and (F), 10 μm; (E) and (G)–(J), 50 μm.

active in some PAN neuroblasts, it is also active in many Ase$^+$ neuroblasts, suggesting that *ttk* transcription is not sufficient for specifying PAN identity (data not shown). Finally, Notch signaling can indirectly repress Ase by downregulating expression of two *ase* activators, the transcription factors Achaete and Scute (Heitzler et al., 1996; Oellers et al., 1994). We favor the idea that Notch signaling mediates Ase repression because inhibiting signaling results in elimination of PAN neuroblast lineages (Figures S7G and S7H). Since all central brain neuroblasts appear to express equal levels of Notch receptor (Figure S8) and report equal levels of signaling through the reporter construct gbe+Su(H) lacZ (data not shown, Almeida and Bray, 2005), other factors must undetectably enhance Notch signaling levels in the PAN neuroblast or otherwise act together with Notch to specify PAN identity.

It is unclear why PAN neuroblasts downregulate Ase, because there are few known Ase target genes. The three neural precursor cell types known to generate neuroblasts—the embryonic neuroepithelium (Jarman et al., 1993), the optic lobe neuroepithelium (Egger et al., 2007), and now the PAN neuroblast—do not express Ase, although the PAN neuroblast is unique in this group for expressing most other neuroblast markers. In the optic lobe, Ase expression is correlated with upregulation of Dap and cell cycle exit (Wallace et al., 2000). This probably does not play a role in the central brain since most of the cycling neuroblasts are Ase$^+$. Ectopic expression of Ase in imaginal discs upregulates *achaete* and the E3 ubiquitin ligase *neuralized* (Brand et al., 1993), but at first glance, neither of these gene products seem like they would conflict with secondary neuroblast fate. Still, Ase expression is clearly not compatible with the production of transit-amplifying lineages because ectopic Ase abolishes them (Figures 1B and 1B′). To test whether the inverse is true and downregulating Ase generates supernumerary transit-amplifying neuroblast lineages, we analyzed *ase*^{1} mutants. Ase protein was gone but additional neuroblasts producing daughters without nuclear Pros were not observed (data not shown). We interpret these results to mean that downregulation of Ase is not an instructive signal for specification of PAN neuroblast identity, but rather a consequence of specification. While failure to express Ase in the secondary neuroblasts might be predicted to lead to

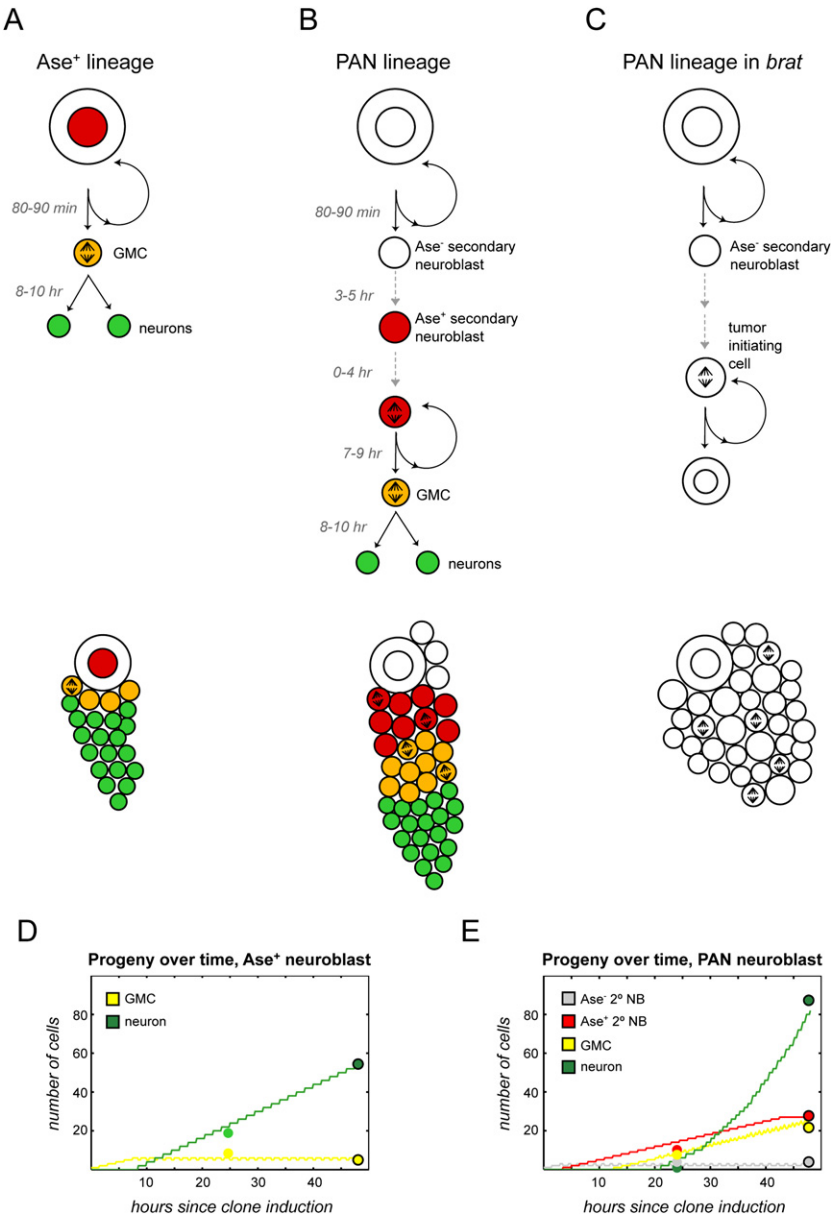

Figure 6. Models of Neuroblast Lineages in the Central Brain

(A–C) Lineage diagrams with estimated time for each stage and cartoons of corresponding neuroblast clones. (A) Self-renewing Ase$^+$ neuroblasts produce progeny that follow the standard GMC-neuron progression. (B) PAN neuroblasts generate Ase$^-$ secondary neuroblasts that mature into self-renewing Ase$^+$ secondary neuroblasts. These transit-amplifying cells produce GMCs, which divide terminally to produce two neurons. (C) In the absence of Brat, Ase$^-$ secondary neuroblasts are unable to become Ase$^+$. Upon mitotic entry, they become tumor-initiating cells.

(D and E) Plots showing production of neuroblast progeny over time. Production of neurons in Ase$^+$ lineages is linear (D), while production of neurons in PAN lineages is exponential (E). Plots were created using Matlab and computer-modeled neuroblast lineages (see Supplemental Experimental Procedures for details). Colored circles correspond to the cell numbers recorded in Table 2.

overgrowth, as in *numb* or *brat*, we could detect no overgrowth in the *ase* mutant brains. This implies that once the PAN neuroblast daughter is born, Ase is not required to promote the transition from immature to mature secondary neuroblast. Nevertheless, Ase expression is a useful reporter of this cell fate transition.

Numb, Brat, Pros, and Transformation of Progenitor Cells

In the PAN lineage, the most recently born daughter cells are Ase$^-$ secondary neuroblasts. This immature state is normally quickly bypassed when the cell becomes an Ase$^+$ secondary neuroblast (Figure 6). Our data indicate that Brat and Numb promote this transition, because in the absence of either protein, the progeny of the PAN neuroblast fail to become Ase$^+$. Numb inhibits Notch signaling, so this phenotype shows that downregulation of Notch allows the secondary neuroblast to mature and become Ase$^+$. Since loss of Brat and loss of Numb have identical phenotypes in 24 hr clones, does this mean that Brat is also an antagonist of Notch? Two points argue against this hypothesis. First, although Brat is expressed in the *Drosophila* sensory organ precursor and segregates asymmetrically (Betschinger et al., 2006), its loss does not cause any change in daughter cell identity (A. Hutterer and J.A.K., unpublished data). This system is sensitive to levels of Notch signaling, so the result is inconsistent with a role for Brat in the regulation of Notch. Second, unlike overexpression of Numb, overexpression of Brat cannot silence reporting through the Notch sensor gbe+Su(H) lacZ in the larval brain (data not shown). Thus, Brat does not seem to regulate cell fate by acting through Notch. While we cannot exclude that Brat regulates Notch signaling in a Su(H)-independent manner, the exact molecular function of Brat in this context remains to be discovered.

Loss of Brat and Numb may cause identical phenotypes in the PAN lineage not because they have identical functions, but because of the nature of the Ase$^-$ secondary neuroblast. In the Ase$^+$ neuroblast lineage, Pros is imported into the nucleus of the neuroblast daughter. One cell fate determinant in addition to Pros is enough to establish GMC fate, because loss of Brat or Numb alone does not prevent neural differentiation (Figures 1G, 1I, 4B, and 4H, and Figure S7A). In the PAN lineage, the Ase$^-$ secondary neuroblast does not have nuclear Pros, so it uses only Brat and Numb to make it different from its mother. For this reason it may be more sensitive to loss of either cell fate determinant. Additionally, besides small size and increased levels of Brat and Numb inherited at the time of its birth, we did not find a single molecular marker that makes the

Ase$^-$ secondary neuroblasts different from PAN neuroblasts. This suggests the Ase$^-$ secondary neuroblast may not yet be strongly committed to its fate. While this unstable state is normally bypassed when the cell becomes an Ase$^+$ secondary neuroblast, in the absence of Brat or Numb, the weak commitment of the Ase$^-$ secondary neuroblast may ensure that no matter which protein is lost, the result will be identical: it reverts to a fate similar to its mother's. Instead of becoming a transient-amplifying progenitor, the Ase$^-$ secondary neuroblast commits to unlimited self-renewal. Because this occurs in a cell that may not express Pros (Figure 5F), mitosis therefore begins the production of cells that are unable to differentiate (Figure 6). This could explain why *brat* neuroblasts do not appear to express Pros or segregate it asymmetrically (Bello et al., 2006; Betschinger et al., 2006), and it could also explain why expressing Pros rescues *brat* tumors. The defect in the lineage caused by *brat*—mitosis in cells not expressing Pros—is now repaired. We propose that blocking full commitment to the intermediate progenitor fate and allowing mitosis in a misspecified daughter may be a general mechanism for causing overgrowth in a transit-amplifying lineage. While transit-amplifying lineages offer the possibility to produce differentiated cells at a faster rate than stem cell lineages without intermediate progenitors (Figures 6D and 6E), this may always come with a higher risk of tumorigenesis. Our data suggest that specification of secondary neuroblast fate is a genetic weak point.

EXPERIMENTAL PROCEDURES

Fly Strains

Fly strains used were 1407-Gal4 inserted in the *insc* promoter (Betschinger et al., 2006); UAS *ase* (Brand et al., 1993); UAS *brat* RNAi (Dietzl et al., 2007); *ase*-Gal4 (Zhu et al., 2006); FRT40A, $brat^{192}$ (Betschinger et al., 2006); FRT40A, $brat^{150}$ (Betschinger et al., 2006); FRT40A, $numb^{15}$ (Berdnik et al., 2002; Bhalerao et al., 2005); FRT82B, $pros^{17}$ (Doe et al., 1991); UAS CD8::GFP (Bloomington stock center); MARCM stocks using C155-Gal4 (Lee et al., 1999); *pros*-Gal4 (a gift from F. Matsuzaki); FRT40A, lgl^1 and FRT40A, lgl^4 (from F. Matsuzaki); FRT82B, $aurA^{87Ac-3}$ and FRT82B, $aurA^{37}$ (Berdnik and Knoblich, 2002); and *UAS* $aPKC^{CAAXWT}$ (Sotillos et al., 2004). To prevent embryonic lethality some UAS constructs were expressed with 1407-Gal4 and $Gal80^{ts}$ (Bloomington stock center 7018) and reared at 18° until larval stages. Then larvae were incubated at 29° for 3 days to allow expression of the transgene. All other transgenes were expressed at 25°.

Antibodies

Antibodies used were guinea pig anti-Ase (affinity purified, 1:100; Bhalerao et al., 2005), mouse anti-Elav (1:100, Developmental Studies Hybridoma Bank, University of Iowa [DSHB]), rat anti-Elav (1:300, DSHB), mouse anti-Pros (1:10, DSHB), guinea pig anti-Dpn (1:1000, gift from J. Skeath), mouse anti-CycE (1:100, H. Richardson), rabbit anti-Mira (1:100; Betschinger et al., 2006), rabbit anti-phospho-histone H3 (1:1000, Upstate), and mouse anti-Insc (1:100; Schaefer et al., 2001).

Immunohistochemistry

To generate MARCM clones, larvae were heat shocked in Eppendorf tubes for 90 min at 37°, then allowed to recover for 24 or 48 hr on fly food at 25°. Other genotypes were dissected as wandering third-instar larvae unless otherwise noted. Larval brains were dissected in PBS, fixed in 5% paraformaldehyde in PBS for 20 min, and blocked using 2% normal donkey serum in PBS with 0.05% Triton X-100. Brains were incubated with primary antibody overnight and labeled using standard methods, then mounted in Vectashield (Vector Laboratories). To visualize cortical actin, we used rhodamine Phalloidin (Molecular Probes) or Alexa 488 Phalloidin (Molecular Probes). Secondary antibodies were conjugated to Alexa 405, Alexa 488, Alexa 568 (all from Molecular Probes), or Cy5 (Jackson Immunofluorescence). Images were recorded on a Zeiss LSM510 confocal microscope.

SUPPLEMENTAL DATA

Supplemental Data include eight figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/14/4/535/DC1/.

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